Effects of Oleamide on Choline Acetyltransferase and Cognitive Activities

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We screened 50 Korean traditional natural plants to measure the activation effect on choline acetyltransferase and attenuation of scopolamine-induced amnesia. The methanolic extracts from Zizyphus jujuba among the tested 50 plants, showed the highest activation effect (34.1% on choline acetyltransferase in vitro). By sequential fractionation of Zizyphus jujuba, the active component was finally identified as cis-9-octadecenoamide (oleamide). After isolation, oleamide showed a 65% activation effect. Administration of oleamide (0.32%) to mice significantly reversed the scopolamine-induced memory and cognitive impairment in the passive avoidance test and Y-maze test. Injection of scopolamine to mice impaired performance on the passive avoidance test (31% decrease in step-through latency), and on the Y-maze test (16% decrease in alternation behavior). In contrast, mice treated with oleamide before scopolamine injection were protected from these changes (12–25% decrease in step-through latency; 1–10% decrease in alternation behavior). These results suggest that oleamide should be a useful chemopreventive agent against Alzheimer’s disease.

Key words: choline acetyltransferase; Zizyphus jujuba; oleamide; Alzheimer’s disease; cognitive impairment

Alzheimer’s Disease (AD) is the most common cause of progressive cognitive dysfunction that affecting approximately four million Americans, causing more than 100000 deaths each year with a total annual cost approaching $70 billion. The reduction of choline acetyltransferase (ChAT, EC 2.3.1.6) activity is related to the degree of dementia and the severity of neuropathological hallmarks of AD. ChAT activity was significantly lower in patients with AD than in age-matched control subjects in frontal cortex, temporal cortex, hippocampus, and cerebellum. As the cognitive dysfunction and other features of AD are mediated by loss of function at cholinergic synapses in the neocortex and hippocampus, agents replacing lost cholinergic function should be useful in the management of this disease.

ChAT catalyzes the synthesis of acetylcholine (ACh) in cholinergic neurons. The enzyme is a single-strand globular protein synthesized in the perikaryon and transported to the nerve terminals probably by both slow and rapid axoplasmic flow. ChAT is present in a high abundance in the cytosol of cholinergic cell bodies. In nerve terminals, about 80–90% is present as a cytosolic protein, a portion of which may be ionically associated with synaptic membranes, whereas the remaining 10–20% of the total enzyme appears to be nonionically bound to the plasma membrane. ChAT is also localized in the cell nucleus, but the mechanisms governing the subcellular distribution of ChAT have not been identified, and the roles that different pools of the enzyme play in regulation of neurotransmitter biosynthesis remain unclear. In spite of the key role of ChAT in neurotransmission, the extremely low amounts and instability of the enzyme in the nervous tissues have limited its biochemical and structural analysis.

One strategy for ameliorating symptoms of AD is the restitution of near normal acetylcholine concentration in the synaptic cleft to improve cholinergic neurotransmission. ChAT activators increased synthesis of ACh to boost the endogenous level of ACh in the brain and thereby to boost cholinergic neurotransmission, resulting in the improvement of cognitive function in mild to moderate AD. In order to find a natural active constituent that has a potent
improving effect on ChAT, we screened 50 herbs and spices. As a result, we found an oleamide (cis-9-octadecenoamide) of *Ziziphus jujuba* (the Rhamnaceae family) which is a traditional Korean food material. *Ziziphus jujuba* is a seed fruit and contains a lot of vitamins, inorganic compounds, and minerals etc. This edible plant has been reported to have the effects of anti-aging and neuronal stabilization by a Korean traditional medical book, Dong-eu-bo-gam, which was written about ethnopharmacological plants.

**Materials and Methods**

*Materials.* The dried plants were purchased from the Kyung-dong herbal market and ground enough to pass through a fine screen (about 1 mm). All chemicals were purchased from Sigma.

*Isolation of oleamide from Ziziphus jujuba.* We screened 50 herbal spices in search for a choline acetyltransferase (ChAT) activator from natural resources. ChAT activators increase cholinergic transmission by improving the enzymatic synthesis of acetylcholine. Among these herbs, edible plants, and spices, the methanol extract from *Ziziphus jujuba* (1 kg) had the greatest effect on ChAT activity (Table 1). The aqueous fraction and the chloroform fraction were obtained from the *Ziziphus jujuba* methanol extract by agitation with a mixture of CHCl₃:H₂O (1:1). The chloroform fraction (5.72 g) of *Ziziphus jujuba* was put successively on a first and second silica gel column (Merck, Darmstadt, Germany; 1 kg) with gradient elution with a mixture of solvents (CHCl₃:MeOH) and a Sep-Pak cartridge (C₁₈ reverse phase; Waters). Analytical HPLC was done using a Waters 2690 pump and controller with a Waters 996 UV detector and Waters µ-bondapack C₁₈ reverse phase (3.9 x 150 mm) column. The data were controlled and analyzed using the Millenium Manager System (Version 2.15; Waters Associates). The data were collected over the range 190–800 nm and the detection was done at 252 nm. Injection of sample was calculated as compared with the control activity. The final concentration of each sample in assay mixture was 250 µg/ml.

*Choline acetyltransferase activity measurement.* Whole cellular extracts were prepared from MC-IXC cells. ChAT activity in MC-IXC cells was measured by a modification of the method of Fonnum.³ For the enzyme source, MC-IXC cells were homogenized in a Glass-Col homogenizer (TERRE-HAUTE, USA) with 5 volumes of homogenizing buffer [20 mM Tris-HCl (pH 7.2), containing 150 mM NaCl, 10 mM MgCl₂, and 0.5% Triton X-100] and centrifuged at 10,000 g-force for 30 min. The resulting supernatant was used as an enzyme source. All extraction steps were done at 4°C. The protein concentration was measured using the BCA kit (Bicinchoninic acid; Sigma Co., St. Louis, USA) with bovine serum albumin (BSA) as the protein standard. The enzymatic reaction contained 0.5% Triton X-100, 200 mM NaCl, 1 mM EDTA, 8 mM choline chloride, 0.1 mM eserine, and [¹⁴C] acetyl-CoA 5 mCi. The ACh formed was extracted with sodium tetraphenylborate in the assay. Radioactivity was measured in a Beckman scintillation counter.

*Passive avoidance performance.* The passive avoidance box was divided into two compartments, one illuminated and one dark, with a grid floor. During the training trial, each mouse was placed in the lighted compartment; as soon as it entered the dark compartment, the door was closed and the mouse received an inescapable shock (0.5 mA, 1 sec). In the testing trial, given 1 day after the training trial, the mouse was again placed in the lighted compart-

<table>
<thead>
<tr>
<th>Plant species used for screening</th>
<th>% activation of ChAT activity¹</th>
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<tbody>
<tr>
<td>Citrus unshiu¹</td>
<td>27.3*</td>
</tr>
<tr>
<td>Allium tuberosum¹</td>
<td>22.5*</td>
</tr>
<tr>
<td>Oenanthe siolonifera¹</td>
<td>30.4*</td>
</tr>
<tr>
<td>Ziziphus jujuba¹</td>
<td>34.1*</td>
</tr>
<tr>
<td>Ziziphus jujuba¹</td>
<td>38.3*</td>
</tr>
</tbody>
</table>

¹ Extracts with methanol.
² Extracts with chloroform.
³ ChAT activity (%): The percentage of enzyme activity value for each sample was calculated as compared with the control activity. The final concentration of each sample in assay mixture was 250 µg/ml.
⁴ Data were significantly different with *P* values < 0.05 when compared with those of control.
Fig. 1. Effects of Individual Components on ChAT Activity of MC-IXC Cells. 

Zizyphus jujuba was fractionated by the first silica gel open column chromatography. Open column: 1.5 × 40 cm, sub-fraction (one bed volume): 52 ml, flow rate: 2.5 ml/min. The column was eluted by the following mixtures three times. The activation of enzyme activity for each fraction was calculated as compared with the control value. Values represent the means (n = 3) ± S.D. Sample concentration was 0.8 mM. Eluants were mixtures of CHCl3 and MeOH [100:0 (1–3), 90:10 (4–6), 80:20 (7–9), 70:30 (10–12), 60:40 (13–15), 50:50 (16–18), 40:60 (19–21), 30:70 (22–24), 20:80 (25–27), 10:90 (28–30), 0:100 (31–33); v/v].

Fig. 2. Effects of Individual Component on ChAT Activity of MC-IXC Cells. 

Zizyphus jujuba, which had higher activity than the others, (≥ 25%) was fractionated by the second silica gel open column chromatography. Open column: 1.5 × 40 cm, sub-fraction (one bed volume): 52 ml, flow rate: 2.5 ml/min. The column was eluted by following mixtures three times. The activation of enzyme activity for each fraction was calculated as compared with the control value. Values represent the means (n = 3) ± S.D. The sample concentration was 0.8 mM. Eluants were mixtures of CHCl3 and MeOH [100:0 (1–3), 90:10 (4–6), 88:12 (7–9), 85:15 (10–12), 83:17 (13–15), 80:20 (16–18), 78:22 (19–21), 75:25 (22–24), 73:27 (25–27), 70:30 (28–30), 0:100 (31–33); v/v].

Statistical analysis. Data were expressed as mean ± S.D. Statistical analysis was done by Student’s t test using Sigma Plot software (Jandel Scientific, California, U.S.A.).

Results and Discussion

Effects of oleamide (cis-9-octadecenoamide) in Zizyphus jujuba on ChAT activity and Isolation of oleamide

We screened 50 Korean traditional natural plants to identify the activation effect of choline acetyltransferase (data not shown). Among these plants, the methanol extract of the ground Zizyphus jujuba with activation effect on ChAT from MC-IXC cell was fractionated by chloroform. The chloroform fraction had the highest activation effect on ChAT activity (Table 1). The chloroform fraction was chromatographed on the first silica gel column yielding 18 new sub-fractions. Among them, No. 6–7 sub-fractions were shown to activate ChAT by more than 32% (Fig. 1). No. 6–7 sub-fractions (90:10 and 80:20) were further chromatographed on a second silica gel column, resulting in 33 new sub-fractions. Among the 33 fractions, No. 6 sub-fraction (third fraction of 90:10) had the highest activation effect on ChAT activity (Fig. 2). The yield was 1.47 g. The fraction with the highest activation effect on ChAT from MC-
IXC cells was put through preparative thin layer chromatography (TLC) (data not shown). Each band was monitored for ChAT activation effect. Between the activation effects of oleamide from *Zizyphus jujuba* and *cis*-9-octadecenoamide (oleamide) (SIGMA) there was no difference (Fig. 3). This compound activated MC-IXC cell ChAT activity in a dose-dependent manner (Fig. 3), confirming that it was the activator for ChAT in *Zizyphus jujuba*.

As shown in Fig. 3, the active component had a single peak at about 252 nm. To identify the structure of this compound, $^1$H/$^13$C-NMR spectral analysis (Fig. 4.B.1, 2) and electron impact mass spectrometry (EI-MS) (Fig. 4.B.3) were done. While low levels of the molecular ion of oleamide can be noted ($m/z$ 281) the predominant fragment peaks were found at lower $m/z$ (59, 72). The structure of oleamide has been reported and we confirmed these results with *cis*-9-octadecenoamide (SIGMA). From these spectral data, the active component was finally identified as oleamide (*cis*-9-octadecenoamide). According to EI-MS, we confirmed that the molecular weight was 281 $m/z$ (unit of mass spectrometry: mass to charge ratios).

**Effect of oleamide on scopolamine-induced memory impairment**

Intraperitoneal injection of scopolamine hydrobromide, which is a muscarinic cholinergic receptor blocker, causes memory deficits and decreases cholinergic activity on behavioral performance, therefore, this method of scopolamine exposure is a useful *in vivo* model for Alzheimer’s disease. In this experiment, mice were treated with oleamide in the drinking water at various concentrations for up to 4 weeks before scopolamine administration. Because the average water intake per mouse per day was approximately 8–10 ml, the amount of oleamide consumed by mice receiving 0.16% oleamide in the drinking water ranged from 14 to 16 mg mouse$^{-1}$ day$^{-1}$. All mice treated with oleamide gained body weight normally (39.3 ± 1.2 g for control mice versus 37.8 ± 1.4 g for mice treated with oleamide for 4 weeks) and did not show any signs of toxicity during the experiment (data not shown).

Then we tested the effects of oleamide on learning and memory *in vivo* using the scopolamine (SCOP)-induced amnesia model. Treatment with SCOP (1 mg/kg), a muscarinic receptor blocker, significantly shortened the latency time (31% decrease in step-through latency) in the retention trial (Fig. 5). However, treatment of mice with oleamide for 3 weeks attenuated the SCOP-induced impairment in a dose-dependent manner (12–25% decreases in step-through latency) with maximal effects observed at a concentration of 0.32%.

Spontaneous alternation behavior, which is regarded as a measure of spatial memory, was investigated using the Y-maze test. Mice injected with SCOP displayed significantly impaired spatial working memory (16% decrease in alternation behavior). Pretreatment with oleamide blunted the SCOP-induced decrease in alternation behavior, with the greatest protection observed in mice pretreated for 3 weeks (Fig. 6A). In contrast, the number of arm entries did not change among all the experimental groups, demonstrating that general locomotor activity was not affected by SCOP (Fig. 6B). In the passive avoidance task and Y-maze task, we used *cis*-9-octadecenoamide (SIGMA) for comparative data and could not detect any differences between oleamide from *Zizyphus jujuba* and *cis*-9-octadecenoamide from SIGMA (data not shown).

In this study, we have shown that oleamide (*cis*-9-octadecenoamide) of *Zizyphus jujuba* was purified from natural edible plants. Oleamide has been reported to be isolated from the cerebrospinal fluid (CSF) of sleep-deprived cats, humans and rats, and shown to cause sleep in rats when injected intravascularly at nanomolar quantities. And oleamide was shown to potentiate 5-hydroxy-tryptamine actions at 5-HT2 receptors, which may in part explain its sleep-inducing properties. However, to our knowledge, this is the first report that oleamide has an activation effect on ChAT. This low-molecular mass material could easily reach the site of action (brain) following oral or transdermal administration because the molecule could cross the blood-brain-barrier, the tight junction controlling the transport of material into the brain. Thus this active component could slow down the decline of cognitive function and memory in some patients with mild or moderate Alzheimer’s disease (AD).
Fig. 4. Structural Analysis of *Zizyphus jujuba*. A: Gradient reversed phase Waters 2690 HPLC analysis of *Zizyphus jujuba*. Oleamide was detected at 24.554 min (CHCl₃:MeOH = 71:29). μ-Bondapack C₁₈ reverse column: 7.8 × 300 mm, mobile phase: an 80-min gradient of 0–100% methanol in water, flow rate: 3.0 ml/min, UV detector: absorbance monitor operating at 252 nm. Injection volume was 40 μl. B.1: ¹H-NMR spectrum. B.2: ¹³C-NMR spectrum. Isolated material was dissolved in methyl-d₃ alcohol-d₃ and recorded on a high resolution NMR spectrometer (Avance DRX 600, Bruker Co., German) operating at 600 MHz and 25°C. B.3. Electron impact mass spectrum of oleamide. The m/z values of the molecular ion and main fragment ions are shown.
Oleamide Improves Antiamnesic Activity
A pathologic hallmark of AD is the formation of senile plaques.\textsuperscript{17} Amyloid-\(\beta\) peptide (A\(\beta\)), a 39–43 amino acid peptide, is a major component of these plaques. A\(\beta\) was shown to have the potential to induce oxidative stress and inflammation in the brain,\textsuperscript{18} which are postulated to play important roles in the pathogenesis of AD.\textsuperscript{19} An intracerebroventricular injection of low nanomolar doses of A\(\beta\)\textsubscript{25–25}\textsuperscript{20} or A\(\beta\)\textsubscript{1–28}\textsuperscript{21} impairs avoidance behavior and Y-maze alternation behavior in mice. Similarly continuous intracerebroventricular infusion of A\(\beta\)\textsubscript{1–40}\textsuperscript{22} or A\(\beta\)\textsubscript{1–42}\textsuperscript{23} induces learning and memory impairment in rats. In this study, we used a scopolamine-induced animal model as an experiment for A\(\beta\)-induced cholinergic impairment. Because scopolamine is a muscarinic cholinergic receptor blocker, it is frequently used to assess the effects of the cholinergic blockade on behavioral performance.\textsuperscript{12} The results presented here were that 3 weeks administration of oleamide effectively protected against scopolamine-induced cholinergic toxicity, and furthermore, decrease of cortical acetylcholine levels results from A\(\beta\)-induced behavioral abnormality.\textsuperscript{22} Therefore oleamide has both ChAT activator and A\(\beta\)-neurotoxicity blocking. In conclusion, oleamide from the natural plant may be a potential chemo-preventive agent against Alzheimer’s disease.

Acknowledgments

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References